Research Article

Evaluation of the Synergistic Antimicrobial Activity of Amikacin with Norfloxacin against *Pseudomonas aeruginosa* Isolated from Buffaloes Clinical Mastitis

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Abstract | Mastitis is a serious and economically problem commonly prevalent in most dairy cattle and buffaloes herds. *Pseudomonas aeruginosa* (*P. aeruginosa*) is opportunistic pathogens involved in veterinary disorders including clinical mastitis in buffaloes. This study aimed to investigate the antibiogram pattern and synergistic effect of amikacin and norfloxacin against resistant *P. aeruginosa* isolates from mastitis origin. In addition, detection of some virulence and antibiotics resistance genes. Two hundred buffaloes were examined and sixty mastitis milk samples were collected from clinical cases from the period from October 2021 until March 2022. The acute mastitis sings were assessed according to cardinal signs of inflammation and milk abnormalities. Out of two hundred buffaloes, sixty (30%) were diagnosed as clinical mastitis according to inflammatory signs and the culture results reveled only 5 (8.3%) were *P. aeruginosa*. Most of *P. aeruginosa* exhibited resistance to most antimicrobials classes. Meanwhile, the minimal inhibitory concentration (MIC) for amikacin and norfloxacin is significantly reduced from 64 µg/mL to 1 µg/mL and from 256 µg/mL to 8µg/mL respectively with frictional inhibitory concentration (FIC) index 0.25. Therefore, the FIC index recognized a synergistic activity between amikacin and norfloxacin against all *P. areuginosa* isolates. The mPCR was an efficient tool for detection of virulence genes (*exo*T, *tox*A, *opr*L, and *isa*I) at 152, 396, 504, 606 bp respectively. In addition, all the *P. aeruginosa* were found to carry the resistance genes (*qnr*S, *qnr*A, *aad*B). The combination of norfloxacin plus amikacin suppressed the resistance pattern *P. aeruginosa* isolates. Therefore, their combination showed synergistic bacterial potential antimicrobial activity in treatment of mastitis due to *P. aeruginosa* infection and help in reducing the resistance problem.

Keywords | Amikacin, *P. areuginosa*, mPCR, Norfloxacin, Virulence, Resistance, Mastitis

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INTRODUCTION

Mastitis is a significant disease of dairy animals particularly buffaloes and caused by numerous infectious and non-infectious determinants causing inflammation of parenchyma of the mammary gland associated with physical, chemical and bacteriological alteration in the milk and pathological alterations in the glandular tissues (Abutarbush, 2010). Mastitis is the most costly and economically disease of highly interest in all dairy herds worldwide. It associated with a substantial drop in milk production, high production costs and low

milk quality (Reetha et al., 2020). Additionally, bovine mastitis constitute a serious risk for animal and human health issue through transmition of zoonotic pathogens and multidrug-resistant bacteria with high levels of antimicrobials resistance (Holko et al., 2019; Oliveira et al., 2022). However, several microbiota were identified from raw milk, subclinical and clinical mastitis in water buffaloes such as *Staphylococcus aureus*, *Streptococcus dysgalactiae, Streptococcus uberis, Bacillus cereus*, *Micrococcus luteus*, *Escherichia coli*, *P. aeruginosa*, and *Citrobacter* species (Baloch et al., 2011). The prevalence of different bacteria in mastitis is frequently varied from area to another according to difference in various determinants factors. In Brazilian study *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus* spp., *Acinetobacter* spp., *P. aeruginosa*, *Shigella flexneri, Streptococcus* spp., *Corynebacterium* spp., and *Escherichia coli* were the common identified pathogens in mastitis (Vásquez-García et al., 2017). Previous studies (Catozzi et al., 2017) reported on the emerging role of Pseudomonas species in subclinical and clinical mastitis. It usually appears in dairy herds as sporadic cases or in outbreaks (Schauer et al., 2021). *P. aeruginosa* is a Gram-negative oxidase-positive rod shape, motile bacterium involved in acute and chronic infections in various species. In ruminants, it had been isolated from cases of clinical and subclinical mastitis in dairy cows, sheep, and goats (Ohnishi et al., 2011; Chen et al., 2018). In a recent study (Petridou et al., 2021) dedicated that *P. aeruginosa* have been isolated from clinical mastitis outbreaks in lactating Holstein cows that exhibited clinical mastitis signs with no responded to ordinary antibiotics treatment. In another study the high prevalence of *P. aeruginosa* in subclinical mastitis as single infections and mixed infections with *Staphylococcus aureus*, *Enterobacter* spp., *Escherichia coli*, *Coagulase negative Staphylococci, Bacillus* spp. (Sumon et al., 2017). The various antibiotics such as ciprofloxacin and cephalothin remain the first effective choice for treatment of mastitis under the veterinary field practices (Gomes and Henriques, 2016). However, the misuse and abuse of antibiotics is strongly related to the development of antibiotic resistance against various antimicrobial groups (Schwarz et al., 2018). The rapid emerging of various resistant *P. aeruginosa* isolates of bovine origin against aminoglycosides antibiotics referred to the presence of the aac(3)-Ib gene (Al-Taee et al., 2019). Furthermore, most strains of *P. aeruginosa* of bovine mastitis contain a type III secretion system that prompt an increase in the number of somatic cells count as well as the biofilms production that reducing the efficacy of antibiotics (Park et al., 2014). Other intrinsic resistance factors such as mutations, acquiring resistance through horizontal gene transfer were harbored by many *Pseudomonas* species and enhanced the development of antimicrobials treatment failures (Cholley et al., 2010). Recently, several molecular techniques are used for detection of different *Pseudomonas* spp. and its virulence determinants with rapid, specific and sensitive pattern (Abdalhamed et al., 2016). The aim of this study was to spot highlights to evaluate the susceptibility and resistance of *P. aeruginosa* and the synergistic effect of amikacin and norfloxacin. In addition to the detection of some virulence and antibiotics resistance genes.

MATERIALS AND METHODS

Study area

Giza is one of the of Egyptian governorates which located in the center of Egypt at 29.26°N 29.67°E. It situated at the west branch of the Nile River opposite to Cairo. It includes a stretch of the left bank of the Nile Valley around Giza, and constitute a large stretch of Egypt's Western Desert. According to population estimates in 2018 the majority of populations are live in urban areas, with 60.9% urbanization rate and the total number of populations was estimated 8,759,000 people and about 5,332,000 people live in urban areas as opposed to only 3,428,000 in rural areas (Wikipedia, 2012).

Animals and samples

Two hundred buffaloes reared in a private farm in Giza governorate were examined and sixty mastitis milk samples were collected from clinical cases of mastitis buffaloes from the period from October 2021 until March 2022. The clinical examination and palpation of udder was done according to (Islam et al., 2012). The acute mastitis sings were assessed according to (fever, inflamed udder andedema, abnormal milk secretion as clotted and flacks milk). All the samples were aseptically collected then transported in a cool condition in icebox at 4°C to the laboratory for further bacteriological examination.

Phenotypic characterization of *P. aeruginosa*

The collected samples were subculture onto specific medium for *P. aeruginosa* (Pseudomonas selective agar, cetrimide agar medium and blood agar) and incubated aerobically at 37 C for 24-48h. The characteristic pure colonies appear as bluish green color and confirmation was carried out based on the pigment production as well as biochemical tests include; oxidase, catalase, coagulase, citrate, OF (Oxidative-Fermentative), urease, Arginine dehydrolase, cetrimide test and nitrate reduction and lipase activity as described by (Quinn et al., 2011).

Antimicrobial suscptability test of *P. aeruginosa* isolated from buffoles

Disk diffusion method was used to test five *P. aeruginosa* isolates against 10 antibiotics of different antimicrobials classes; (Oxoid Ltd., Basingstoke, UK): 100 IU penicillin, 20/10 µg, amoxicillin/clavulanic acid, 30µg cefotaxime, 10 µg norfloxacin, 30 µg chloramphenicol, 30 µg tetracycline, 10

µg gentamicin, 100 µg spectinomycin, 30 µg streptomycin, 30µg amikacin and 1.25/23.75 µg sulfamethoxazole/ trimethoprim. A suspension of the bacterial suspension was prepared to equal the turbidity of a 0.5 McFarland standard (1.5 × 108 colony forming units (CFU) ml–1). The results was determined as resistance, sensitive, and intermediate according to (CLSI, 2018).

Determination of the combined effect between amikacin and norfloxacin against resistant *P. aeruginosa* by checker broad dilution assay

This test is a standard technique used to determine the synergism effect between two antibiotics and as done using of the checker broad dilution assay and estimation the FIC index as described by (Bellio et al., 2021).

Molecular detection of *P. aeruginosa* virulence associated genes

DNA extraction. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from Metabion (Germany) are listed in Table 1 as described previously by the following authors (Frana et al., 2001; Matar et al., 2002; Xu et al., 2004; Winstanley et al., 2005; Robicsek et al., 2006; Bratu et al., 2006).

For mPCR, Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. For mPCR, primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 13 µl of water, and 8 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

The PCR products were electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. Followed by gel analysis, 40 µl of the mPCR products were loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo) and gelpilot 100 bp plus ladder (Qiagen, gmbh, Germany) were used to determine the fragment size and photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RESULTS

Prevalence of *P. aeruginosa* from clinical mastitis in buffaloes

In the current study, out of 200 examined buffaloes, 60 (30%) mastitic milk were collected and then bacteriologically examined. The results revealed that *P. aeruginosa* was

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions of virulence and antibiotics resistance genes of *P. areuginosa*.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
toxA	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 40 sec.	72°C 10 min.	Matar *et al.*, 2002
exoT	AATCGCCGTCCAACTGCATGCG TGTTCGCCGAGGTACTGCTC	152	94°C 5 min.	94°C 30 sec.	58°C 30 sec.	72°C 30 sec.	72°C 7 min.	Winstanley *et al.*, 2005
exoS	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	118	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min.	
oprL	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504	94°C 5 min.	94°C 30 sec.	55°C 40 sec	72°C 45 sec.	72°C 10 min	Xu *et al.*, 2004
lasI	ATGATCGTACAAATTGGTCGGC GTCATGAAACCGCCAGTCG	606	94°C 5 min.	94°C 30 sec.	56°C 40 sec	72°C 45 sec.	72°C 10 min	Bratu *et al.*, 2006
qnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516	94°C 5 min.	94°C 30 sec.	55°C 40 sec	72°C 45 sec.	72°C 10 min	Robicsek *et al.*, 2006
qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	94°C 5 min	94°C 30 sec.	55°C 40 sec	72°C 40 sec.	72°C 10 min.	
aadB	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	319	94°C 5 min.	94°C 30 sec.	58°C 30 sec	72°C 30 sec	72°C 7 min	Frana *et al.*, 2001

prevalent with five (8.3%) as showed in Table 2. The characteristic colony of *P. aeruginosa* on cetrimide agar medium appeared as bright green colonies as showed in Figure 1. After the isolation of *P. aeruginosa* isolates, the biochemical identification and enzymatic activities were done as described in Table 3. All the tested isolates showed positive reaction for catalase, oxidase, citrate, nitrate reduction as well as produce pigment, gelatin hydrolysis and positive for cetrimide test. Concerning to enzymatic activity, all isolates were positive only for arginine dehydrolysis and lipase activity. For oxidative fermentation, the isolates were positive only for mannitol sugar and negative for other tested sugars.

Table 2: Prevalence of *P. aeruginosa* recovered from clinical mastitis in buffaloes.

P. aeruginosa		Mastitis animals		Total examined
%	Nu	%	Nu	200
8.33	5	30	60	

Figure 1: Bright green color colonies of *P. aeruginosa* on cetrimide agar medium.

Results of the antibiogram profile of *P. aeruginosa*

Regarding to the antimicrobial resistance profile and the MIC for five purified *P. aeruginosa* isolates isolated from clinical mastitis. Ten antibiotics belonging to six antimicrobial classes were used in the analysis. The most of *P. aeruginosa* isolates showed multidrug resistance to all type of antibiotics as recorded in Table 4 and Figure 2. In addition, the checkerboard dilution assay was applied as a specific and standard method applied to evaluate the synergistic effect between two antibiotics and applied in a 96-well microplate and FIC was measured to find the type of interaction between the two antibiotics. Our results indicated the synergistic activity between amikacin and norfloxacin against *P. aeruginosa* isolates with FIC index ranging from 0.18 to 0.5. Moreover, the MIC for amikacin and norfloxacin is significantly reduced from 64 µg/mL to 1 µg/mL and from 256 µg/mL to 8µg/mL respectively with FIC index 0.25 as showed in Table 5.

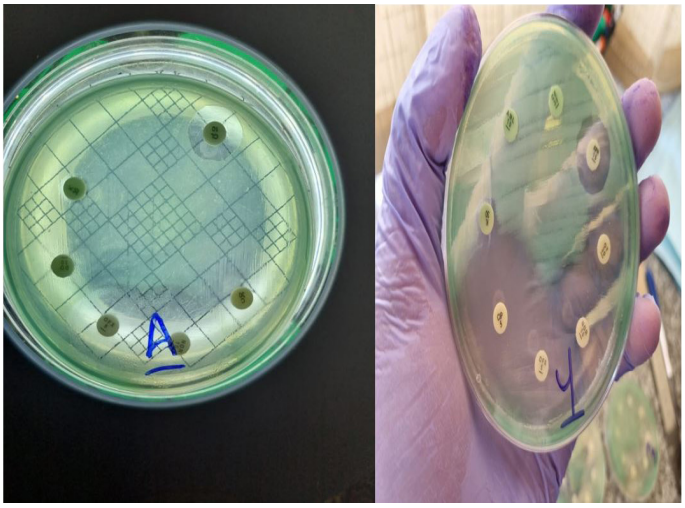

Figure 2: Sensitivity test of *P. aeruginosa* against different antimicrobials with synergism profile.

Molecular detection of virulence and antibiotics resistance gene of *P. aeruginosa*

The mPCR was used efficiently for the detection of some virulence genes of *P. aeruginosa* isolates through successful amplification of *exo*T, *tox*A, *opr*L, and *ias*I genes at 152, 396, 504, 606 bp, respectively as well as the antibiotics resistant genes for aminoglycosides and fluoroquinolones antibiotics; *qnr*S, *qnr*A and *aad*B at 417, 516 and 319 bp, respectively as in Figure 3-5.

Table 3: Biochemical tests and enzymatic activity of *P. aeruginosa* recovered from clinical mastitis in buffaloes.

Cetrimide Test	Pigment	Gelatin hydrolysis	NR	H2S	Citrate	Urease	VP	MR	Indole	Coagulase	Oxidase	Catalase
+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Enzymatic activity								Oxidative Fermentation				
Alkaline phosphatase	Lecithinase	Lysine	Lipase	Arginine dehydrolase	Sucrose	Sorbitol		Glucose	Inulin	Maltose	Lactose	Mannitol
-ve	-ve	-ve	+ve	+ve	-ve	-ve		-ve	-ve	-ve	-ve	+ve

NR: nitrate reduction; Vp: Voges-Proskauer; MR: Methyl red.

Table 4: Results of the antimicrobial susceptibility pattern of the *P. areuginosa* from clinical mastitis in buffaloes.

Antibiotic	Class	R		M		S	
		Nu	%	Nu	%	Nu	%
Penicillin,	β lactams	5	100	0	0	0	0
Amoxicillin/ clavulanic acid	β lactams	4	80	1	20	0	0
Cefotaxime	β lactams	3	60	2	40	0	0
Norfloxacin	fluoroquinolone	2	40	1	20	2	40
Streptomycin,	Aminoglycosides	3	60	2	40	0	0
Amikacin	Aminoglycosides	1	20	1	20	3	60
Gentamicin	Aminoglycosides	2	40	2	40	1	20
Spectinomycin,	Aminoglycosides	4	80	0	0	1	20
Chloramphenicol	phenicol,	4	80	1	20	0	0
Tetracycline,	Tetracycline,	3	60	1	20	1	20
Sulfamethoxazole/ trimethoprim	sulphonamides	3	60	1	20	0	0

R: resistance; M: moderate; S: sensitive

Table 5: The MIC for amikacin and norfloxacin combination and FIC index against *P. areuginosa* by the checker broad assay.

	EFIC	FIC NF	FIC Ak	Mic NF in comb	Mic Ak in comb	Mic NF	Mic Ak	Iso-late
Synergism	0.18	0.125	0.6	32	2	256	32	1
Synergism	0.5	0.25	0.25	2	0.5	8	1	2
Synergism	0.5	0.25	0.25	32	16	128	64	3
Synergism	0.5	0.25	0.25	8	16	32	64	4
Synergism	0.18	0.125	0.25	16	16	128	64	5

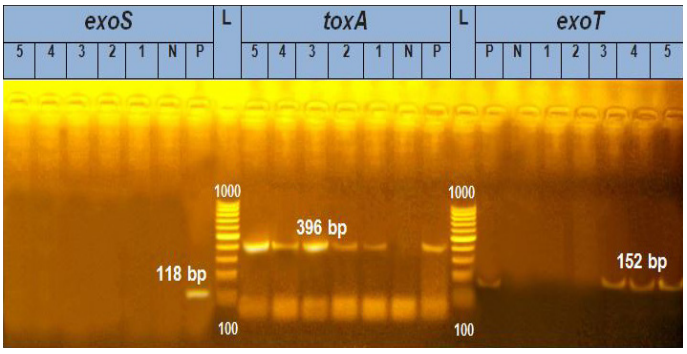

Figure 3: Amplification of *tox A, exo S* and *exo T* genes of *P. aeruginosa* at 396bp, 118 bp and 152 bp respectively, by multiplex PCR. Lane L: 100 bp DNA ladder; Lane 3–5 positive samples for exoT at 152 bp; and 1-5 for toxA genes at 396 bp with no detection of exoS; Lane N: control negative; Lane P: control positive.

DISCUSSION

Mastitis is a major serious and financial disease affecting dairy herds worldwide causing significantly drop in milk production and quality, high veterinary and treatment costs with the probability of transmission of some zoonotic diseases and antibiotics residue through milk (Abdalhamed et al., 2016; Kibebew, 2017). In veterinary practices, *P. aeruginosa* usually associated with several sporadic disorders including urinary tract infection, chronic pyodermia, and dermatitis and otitis media besides the intramammary infections that resulted from soil or environmental contamination (Schauer et al., 2021). In last years, *P. aeruginosa* contribute a serious problem in both large dairy herds as well as small farmers as serious cause of clinical mastitis with significant economic losses among (Klaas and Zadoks, 2018).

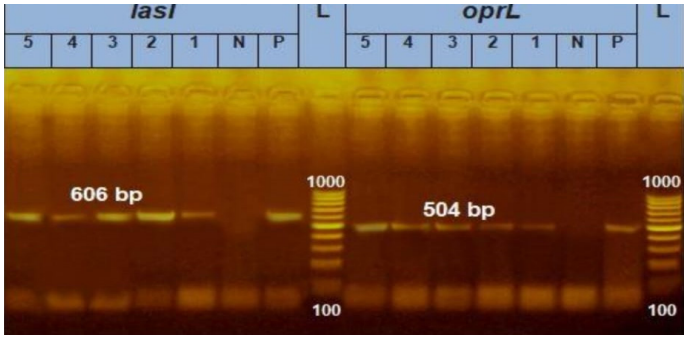

Figure 4: Amplification of *iasI and oprL* genes of *P. aeruginosa* at 606 and 504 bp by multiplex PCR. Lane L: 100 bp DNA ladder; Lane 1–5 positive samples. Lane N: Control Negative; Lane P: Control Positive.

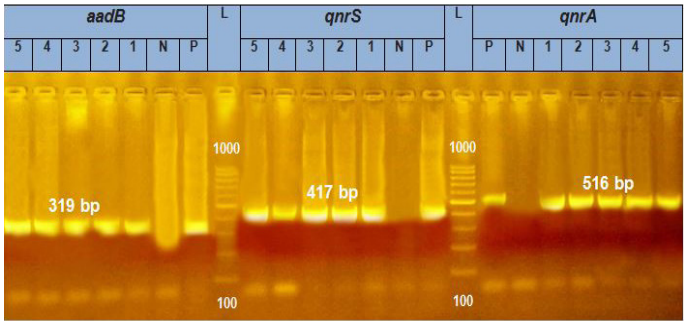

Figure 5: Amplification of *aadB, qnrS* and *qnrA* genes of *P. aeruginosa* at 319bp, 417 bp and 516 bp, respectively, by multiplex PCR. Lane L: 100 bp DNA ladder; Lane 1–5 positive samples for *aadB, qnrS* and *qnrA* genes; Lane N: control negative; Lane P: control positive.

In our study, examination of 200 buffaloes, 60 (30%) were diagnosed as clinical mastitis based on the clinical features of udder and milk as well as bacteriological culturing revealed that *P. aeruginosa* was prevalent with five (8.3%). Nearly similar finding was reported in Egypt by (El-Shafii, 2016) who identified twenty isolates of *P. aeruginosa* (7.4%) out of 270 milk samples from private and governmental cattle farms in three provinces (130 Dakahlia, 66 Sharkia and 74 Damieta) governorates. In a comparative study, in dairy lactating Holstein cows, 40(23.5%) out of 170 animals were observed as clinical mastitis and *P. aeruginosa*

was prevalent with higher prevalence rate 15(37.5%) (Petridou et al., 2021). Furthermore, higher prevalence of *P. aeruginosa* 37 (16.8%) was reported by (Al-Ruaby et al., 2022) from 220 small holder bovine milk samples from different districts in Iraq. On the other hand, lower prevalence rate of *P. aeruginosa* 5.4% was recorded in South Bengal in bovine with subclinical mastitis (Banerjee et al., 2017). The variation between various studies in the prevalence of *P. aeruginosa* may attributed to difference in the geographic area, hygienic practices, environmental contamination, bacterial count in the studied area as well as the predominant of microorganisms in the environment.

After the isolation of *P. aeruginosa* isolates, the biochemical identification and enzymatic activities were performed. All the tested isolates showed positive reaction for catalase, oxidase, citrate, nitrate reduction as well as produce pigment, gelatin hydrolysis and positive for cetrimide test. In addition, all isolates were positive only for arginine dehydrolysis and lipase activity. For oxidative fermentation, the isolates were positive only for mannitol sugar and negative for other tested sugars. Similar results were previously described by (Carter and Wise, 2004; Al-Taee et al., 2019; Reetha et al., 2020) reported that 16 *P. aeruginosa* biotypes utilized galactose, mannose, and mannitol and displayed a green pigment and showed β hemolysis on blood agar with colony of green tinged with metallic sheen on cetrimide media. Furthermore, (Quinn et al., 2015; Al-Ruaby et al., 2022) applied the analytical profile index (API-20E) for biochemical testing of six *P. aeruginosa* isolates recovered from bovine mastitis. Additionally, (Sekhi, 2021) recorded that all the tested *P. aeruginosa* produce haemolysin, phospholipase while 54.28% of tested isolates give the lecithinase activity and 34.28% produce alkaline protease. Therefore, the biochemical activity of *P. aeruginosa* isolates is an indicator for its ability to yield virulence factors.

Regarding to the antimicrobial resistance profile and the MIC for five purified *P. aeruginosa* isolates isolated from clinical mastitis. A total of 10 antibiotics belonging to six antimicrobial classes were used in the analysis. The most of *P. aeruginosa* isolates showed multidrug resistance to all type of antibiotics as recorded in Table 4. This was in contact with a similar study in Egypt (El-Shafii, 2016) reported that the most *P. aeruginosa* isolates showed multidrug resistance to 17 types of antibiotic with resistance rate 25%-100%. The tested isolates showed resistance for quinolone, aminoglycosides, polypeptides sulphonamides, Lincomides, and β lactams groups. The high resistance for ofloxacin enrofloxacin, nalidixic acid and ciprofloxacin with (95%, 85%, 85% and 80%, respectively), while for neomycin, amikacin and gentamycin was 70%, 65%, and 50% respectively. Nearly resistance rate (85-95%) was nearly observed for colstin sulfate and sulfa methaxozoletrimethoprim, pencillin, ampicillin, tetracycline, chloramphenicol, lincomycin and clindamycin. Furthermore, (Al-Taee et al., 2019) demonstrated that most *P. aeruginosa* isolates exhibited multidrug resistance rate ranged from 25%-100% to different 17 antibiotics types. Moreover, (Sekhi, 2021) tested 35 *P. aeruginosa* for various antibiotics types and all isolates reported 100% resistance to ampicillin, oxacillin with higher resistance to other antibiotics cefotamine, impenem, ticarcillin (71.42%, 57.14%, 37.14%), respectively. Meanwhile, a different study conducted in Iraq, by (Al-Ruaby et al., 2022) revealed that Ciprofloxacin, amikacin and Norfloxacin, were the most effective antibiotics against *P. aeruginosa* isolates while completely resistant to erythromycin and tetracycline

The drug combination has become a new trend and a key point to reduce the resistance evolved by many bacterial species. The checkerboard dilution assay was applied as a specific and standard method to evaluate the synergistic effect between two antibiotics and applied in a 96-well microplate and FIC was measured to find the type of interaction between the two antibiotics (El-Demerdash and Bakry, 2020; Bellio et al., 2021). Our results indicated the synergistic activity between amikacin and norfloxacin against *P. aeruginosa* isolates with FIC index ranging from 0.18 to 0.5. Moreover, the MIC for amikacin and norfloxacin is significantly reduced from 64 µg/mL to 1 µg/mL and from 256 µg/mL to 8µg/mL, respectively with FIC index 0.25. This was in contact with (Farhan et al., 2021) pronounced the synergism and the antimicrobial activity between Imipenem and amikacin in the treatment of multi drug resistant *P. aeruginosa* isolates.

Traditional PCR and quantitative real-time PCR practices were efficiently applied for the detection of *P. aeruginosa* harbored the resistance genes *bla* IMP and *aac*(6)-Ib as well as genotyping of *P. aeruginosa* isolates may be grouped with multiple locus variable-number-tandem repeat analysis (MLVA) to determine the relationship and similarities between strains (Farhan et al., 2021; Schauer et al., 2021). Our study applied mPCR for the detection of some virulence genes of *P. aeruginosa* isolates through successful amplification of *exo*T, *tox*A, *opr*L, and *ias*I genes at 152, 396, 504, 606 bp respectively as in Figures 3 and 4. In addition, antibiotics resistant genes for aminoglycosides and fluoroquinolones antibiotics; *qnr*S, *qnr*A and *aad*B were successfully detected at 417, 516 and 319 bp, respectively as in showed in Figure 5. Other similar studies described the detection of several virulence determinants genes by PCR such as (Sekhi, 2021) identified that out of 35 *P. aeruginosa* isolates, the *tox*A and *exo*S genes were found in 35(100%) and 19 (54.23%), respectively. In a comparative study in Morocco, the *P. aeruginosa* isolates recovered from clinical specimens found to carry the *las*B and *exo*S virulence genes with the same percentage (98.7%) (Elmouaden et al., 2019). In a similar study in cattle in South Bengal

with subclinical mastitis *P. aeruginosa* isolates harbored virulence genes such as *tox*A (63.2%) and *exo*S (36.8%) (Banerjee et al., 2017).

Our results indicated that most of the *P. aeruginosa* strains had carried the resistance genes (*qnr*S, *qnr*A, *aad*B) as showed in Figure 5. This in agreement with (Pang et al., 2019) who suggested that the resistance pattern of *P. aeruginosa* to several antimicrobials had been described either due to the intrinsic acquire new resistance mechanisms, which resulting in the emergence of multidrug resistant *P. aeruginosa* strains. Thus, acquired resistance occurs through chromosomal mutations or through the acquisition of resistance genes across horizontal transfer especially for fluoroquinolones, carbapenems, and aminoglycosides groups. On the same viewpoint, (Ahmed, 2022) concluded that multidrug resistant *P. aeruginosa* strains had the highest acquisition of antimicrobials resistance genes particular against aminoglycoside, β-lactam, fluoroquinolones, fosfomycin, sulfonamides, and phenicol.

CONCLUSIONS AND RECOMMENDATIONS

P. aeruginosa is opportunistic pathogens and one of the causative agents for clinical mastitis in buffaloes. In addition, *P. aeruginosa* isolates showed high resistance rate to the most antibiotics classes. Moreover, the use of both amikacin and norfloxacin had potential antimicrobial synergistic activity against the multidrug resistant *P. aeruginosa* isolates from mastitis and suppress its resistance pattern. The mPCR was efficient tool for detection of virulence genes (*exo*T, *tox*A, *opr*L, and *isa*I). In addition, all the *P. aeruginosa* were found to carry the resistance genes (*qnr*S, *qnr*A, *aad*B).

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NOVELTY STATEMENT

The aim of our study was to determine the prevalence and antimicrobial resistance pattern of *Pseudomonas aeruginosa* from mastitis in buffaloes and the synergistic effect of some antibiotics against the resistant strains. In addition, to application of mPCR technique for identification of virulence and antibiotics resistance genes among isolated *Pseudomonas aeruginosa.*

AUTHOR'S CONTRIBUTION

Conceptualization: HMA, WSM, SSM, RSE and HEN. Methodology: HMA, WSM, SSM, RSE and HEN. Formal analysis: HMA, WSM and SSM. Investigation: HMA, WSM, SSM, RSE and HEN. Resources: HMA, WSM, SSM, RSE and HEN. Data curation: HMA and WSM. Writing original draft preparation: All authors. WSM: writing, review and editing. All authors have read and agreed to the published version of the manuscript.

Ethical approval

This study was performed according to the rules and regulations of the Faculty of Veterinary Medicine, the University of Sadat City, Egypt (Approval no. VUSC-014-1-22). This study followed the guidelines of the ethics committee and current legislation on research and ethical approval of the Faculty of Veterinary Medicine (Local ethical approval), University of Sadat City, Egypt.

Data availability

All authors agree that the data presented in this study are openly available through the Advances in Animal and Veterinary Sciences journal platform without any restrictions.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Abdalhamed A, Zeedan GG, Hussein H, Abdeen E (2016). Molecular and biochemical characterization of pseudomonas species isolated from subclinical mastitis milk and ice cream and its susceptibility to allium sativum and commiphorra molmol plant extracts in Egypt. Int. J. Adv. Res., 4(8): 1669–1679. https://doi.org/10.21474/IJAR01/1378

Abutarbush SM (2010). Veterinary medicine. A textbook of the diseases of cattle, horses, sheep, pigs and goats, 10th edition. Can. Vet. J., 51(5): 541.

Ahmed OB (2022). Detection of antibiotic resistance genes in *Pseudomonas aeruginosa* by whole genome sequencing. Infect. Drug Resist., 15: 6703–6709. https://doi.org/10.2147/IDR.S389959

Al-Ruaby KJ, Mohammed MI, Touhali IS (2022). Study synergistic interactions between antibiotics and ascorbic acid against *Pseudomonas aeruginosa* isolated bovine mastitis. Red. Vet. Rev. Electron. Vet., 23(3): 119-129. http://www.veterinaria .org

Al-Taee HSR, Al-Samarraae IAA, Al-Ahmed HI (2019). Antibiotic susceptibility and molecular detection of *Pseudomonas aeruginosa* isolated from bovine mastitis. Iraq. J. Vet. Med., 43(2): 77–85. https://doi.org/10.30539/iraqijvm.v43i2.536

Baloch H, Rind R, Kalhoro DH, Kalhoro AB (2011). Study on the incidence of clinical mastitis in buffaloes caused by bacterial species. Pak. J. Agric. Agric. Eng. Vet. Sci., 27(1): 83-93.

Banerjee S, Batabyal K, Joardar SN, Isore DP, Dey S, Samanta I, Samanta TK, Murmu S (2017). Detection and characterization of pathogenic *Pseudomonas aeruginosa* from bovine subclinical mastitis in West Bengal, India. Vet. World, 10(7): 738–742. https://doi.org/10.14202/vetworld.2017.738-742

Bellio P, Fagnani L, Nazzicone L, Celenza G (2021). New and simplified method for drug combination studies by checkerboard assay. Methods, 8(July): 101543. https://doi.org/10.1016/j.mex.2021.101543

Bratu S, Gupta J, Quale J (2006). Expression of the las and rhl quorum-sensing systems in clinical isolates of *Pseudomonas aeruginosa* does not correlate with efflux pump expression or antimicrobial resistance. J. Antimicob. Chem., 58(6): 1250–1253. https://doi.org/10.1093/jac/dkl407

Carter GR, Wise DJ (2004). Essentials of veterinary bacteriology and mycology. 6th ed. Iowa: The Iowa State Press. pp. 125–126.

Catozzi C, Sanchez Bonastre A, Francino O, Lecchi C, De Carlo E, Vecchio V, Martucciello A,Fraulo P, Bronzo V, Cuscó A, D'Andreano S,Ceciliani F (2017). The microbiota of water buffalo milk during mastitis. PLoS One, 12(9): 1–20. https://doi.org/10.1371/journal.pone.0184710

Chen JW, LauYY, Krishnan T, Chan KG, Chang CY (2018). Recent advances in molecular diagnosis of *pseudomonas aeruginosa* infection by state of the art genotyping techniques. Front. Microbiol., 9: 1104. https://doi.org/10.3389/fmicb.2018.01104

Cholley P, Gbaguidi-Haore H, Bertrand X, Thouverez M, Plésiat P, Hocquet D, Talon D (2010). Molecular epidemiology of multidrug-resistant *Pseudomonas aeruginosa* in a French university hospital. J. Hosp. Infect., 76(4): 316–319. https://doi.org/10.1016/j.jhin.2010.06.007

CLSI (2018). 13th Edition: Performance standards for antimicrobial disk susceptibility tests. www.clsi.org.

El-Demerdash S, Bakry N (2020). Evaluation of the synergistic effect of amikacin with cefotaxime agains *Pseudomonas aeruginosa* and its biofilm genes expression. Gene Exp. Phenotypic Traits. https://doi.org/10.5772/intechopen.91146

Elmouaden C, Laglaoui A, Ennanei L, Bakkali M, Abid M (2019). Virulence genes and antibiotic resistance of *Pseudomonas aeruginosa* isolated from patients in the Northwestern of Morocco. J. Infect. Dev. Countr. 13(10): 892–898. https://doi.org/10.3855/jidc.10675

El-Shafii SA (2016). Detection of multidrug resistance genes in *Pseudomonas aeruginosa* isolated from bovine mastitic milk. J. Dairy. Vet. Anim. Res., 3(2): 43–49. https://doi.org/10.15406/jdvar.2016.03.00071

Farhan SM, Raafat M, Abourehab MA S, El-Baky RMA, Abdalla S, El-Gendy AO, Azmy AF (2021). Effect of Imipenem and Amikacin Combination against Multi-Drug Resistant *Pseudomonas aeruginosa*. Antibiotics, 10(11): 1–13. https://doi.org/10.3390/antibiotics10111429

Frana T S, Carlson SA, Griffith RW (2001). Relative distribution and conservation of genes encoding aminoglycoside-modifying enzymes in *Salmonella enterica* serotype typhimurium phage type DT104. Appl. Environ. Microbiol., 67(1): 445–448. https://doi.org/10.1128/AEM.67.1.445-448.2001

Gomes F, Henriques M (2016). Control of bovine mastitis: Old and recent therapeutic approaches. Curr. Microbiol., 72(4): 377–382. https://doi.org/10.1007/s00284-015-0958-8

Holko I, Tančin V, Vršková M, Tvarožková K (2019). Prevalence and antimicrobial susceptibility of udder pathogens isolated from dairy cows in Slovakia. J. Dairy Res., 86: 436–439. https://doi.org/10.1017/S0022029919000694

Islam M, Islam M, Islam M, Rahman M, Islam M (2012). Prevalence of subclinical mastitis in dairy cows in selected areas of Bangladesh. Bang. J.Vet. Med., 9(1): 73–78. https://doi.org/10.3329/bjvm.v9i1.11216

Kibebew K (2017). Bovine mastitis: A review of causes and epidemiological point of view. J. Bio. Agric. Health, 7(2): 1–14. www.iiste.org

Klaas IC, Zadoks RN, (2018). An update on environmental mastitis: Challenging perceptions. Transbound. Emerg. Dis. 65(Suppl1): 166–185. https://doi.org/10.1111/tbed.12704

Matar GM, Ramlawi F, Hijazi N, Khneisser I, Abdelnoor AM (2002). Transcription levels of *Pseudomonas aeruginosa* exotoxin A gene and severity of symptoms in patients with otitis externa. Curr. Microbiol. 45(5): 350–354. https://doi.org/10.1007/s00284-002-3703-z

Ohnishi M, Sawada T, Hirose K, Sato R, Hayashimoto M, Hata E Yonezawa C, Kato H (2011). Antimicrobial susceptibilities and bacteriological characteristics of bovine *Pseudomonas aeruginosa* and *Serratia marcescens* isolates from Mastitis. Vet. Microbiol., 154: 202–220. https://doi.org/10.1016/j.vetmic.2011.06.023

Oliveira RP, Aragão BB, PimenteR, de Melo B, da Silva DMS, Carvalho, RG, Juliano MA, Farias MPO, Lira NSC, Mota RA (2022). Bovine mastitis in northeastern Brazil: Occurrence of emergent bacteria and their phenotypic and genotypic profile of antimicrobial resistance. Comp. Immunol. Microbiol. Infect. Dis., 85: 101802. https://doi.org/10.1016/j.cimid.2022.101802

Pang Z, Raudonis, R, Glick BR, Lin TJ, Chen Z (2019). Antibiotic resistance in *Pseudomonas aeruginosa:* Mechanisms and alternative therapeutic strategies. Biotech. Adv., 37: 177192. https://doi.org/10.1016/j.biotechadv.2018.11.013

Park AJ, Surette MD,Khursigara CM, (2014). Antimicrobial targets localize to the extracellular vesicle-associated proteome of Pseudomonas aeruginosa grown in a biofilm. Front. Microbiol. Sec. Antimicrobials, Resistance and Chemotherapy. 5.464:1-14. https://doi.org/10.3389/fmicb.2014.00464

Petridou EJ, Fragkou IA, Lafi SQ, Giadinis ND (2021). Outbreak of *Pseudomonas aeruginosa* mastitis in a dairy cow herd in northern Greece and its control with an autogenous vaccine. Pol. J. Vet. Sci., 24(2): 303–305.

Quinn PJ, Markey BK, Leonard FC, Fitz Patrick ES, Fanning S, Hartigan PJ (2011). Veterinary Microbiology and Microbial Diseases. 2nd ed. Ames, IA: Blackwell Publishing Ltd. pp. 287–290.

Reetha TL, Manickam R, Puvarajan B (2020). PCR Based Detection of *Pseudomonas aeruginosa* in Mastitic Cow Milk. International J. Curr. Microbiol. Appl. Sci., 9(2): 194–199. https://doi.org/10.20546/ijcmas.2020.902.024

Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. (2006). Fluoroquinolone-modifying enzyme: A new adaptation of a common aminoglycoside acetyltransferase. Nat. Med., 12(1): 83–88. https://doi.org/10.1038/nm1347

Schauer B, Wald R, Urbantke V, Loncaric I, Baumgartner M (2021). Tracing mastitis pathogens epidemiological investigations of a *pseudomonas aeruginos*a mastitis outbreak

in an Austrian dairy herd. Animals, 11(2): 1–11. https://doi.org/10.3390/ani11020279

Schwarz S, Feßler AT, Loncaric I, Wu C, Kadlec K, Wang Y, Shen J (2018). Antimicrobial Resistance among Staphylococci of Animal Origin. In Microbiol. Spect., 6(4): https://doi.org/10.1128/microbiolspec.ARBA-0010-2017

Sekhi RJ (2021). Isolation and identification of pathogenic *Pseudomonas aeruginosa* from cow mastitis and detect some virulence factor using specific genetic marker. J. Educ. Pur. Sci. Univ. Thi-Qar., 11(1): 1–9.

Sumon S MMR, Ehsan MA, Islam MT (2017). Subclinical mastitis in dairy cows: Somatic cell counts and associated bacteria in Mymensingh, Bangladesh. J. Bang. Agric. Univ., 15(2): 266–271. https://doi.org/10.3329/jbau.v15i2.35073

Vásquez-García A, Silva TS, de AlmeidaQueiroz SR, Godoy SHS, Fernandes AM, Sousa RLM, Franzolin R. (2017). Species identification and antimicrobial susceptibility profile of bacteria causing subclinical mastitis in buffalo. Pesq. Vet. Bras. 37(5):447-452. DOI: 10.1590/S0100-736X2017000500004

Wikipedia (2012). Giza governorate. http://www.giza.gov.eg

Winstanley C, Kaye SB, Neal TJ, Chilton HJ, Miksch S, Hart CA (2005). Genotypic and phenotypic characteristics of *Pseudomonas aeruginosa* isolates associated with ulcerative keratitis. J. Med. Microbiol., 54(6): 519–526. https://doi.org/10.1099/jmm.0.46005-0

Xu J, Moore JE, Murphy PG, Millar BC, Elborn JS (2004). Early detection of *Pseudomonas aeruginosa* comparison of conventional versus molecular (PCR) detection directly from adult patients with cystic fibrosis (CF). Ann. Clin. Microbiol. Antimicrob., 3: 1–5. https://doi.org/10.1186/1476-0711-3-21